Functional characterization of a novel desaturase from *Physcomitrella patens* (Hedw.) B.S.G.

Doctoral thesis
in the biology faculty
of Hamburg University

Submitted by
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Hamburg

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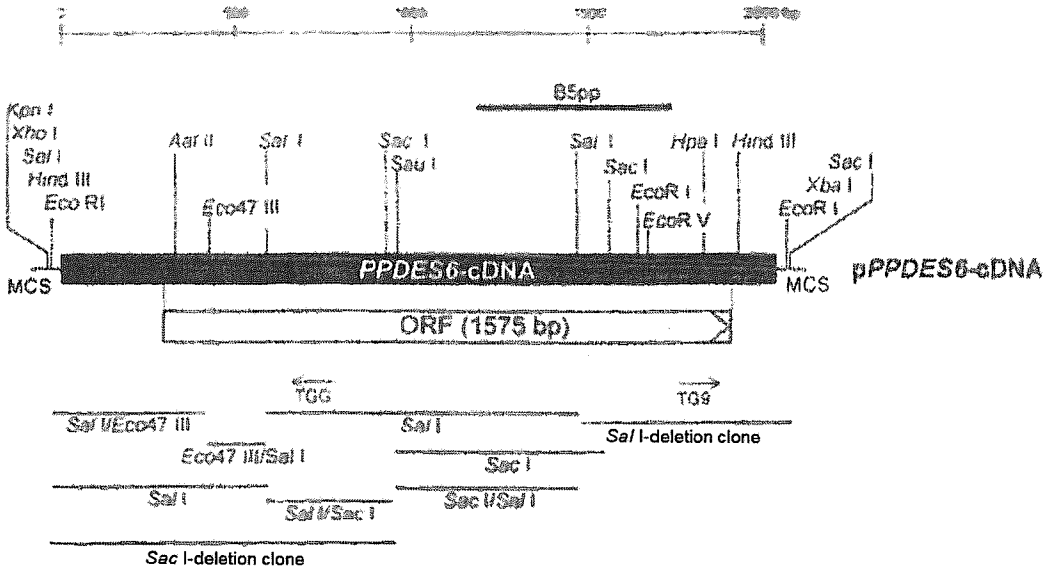
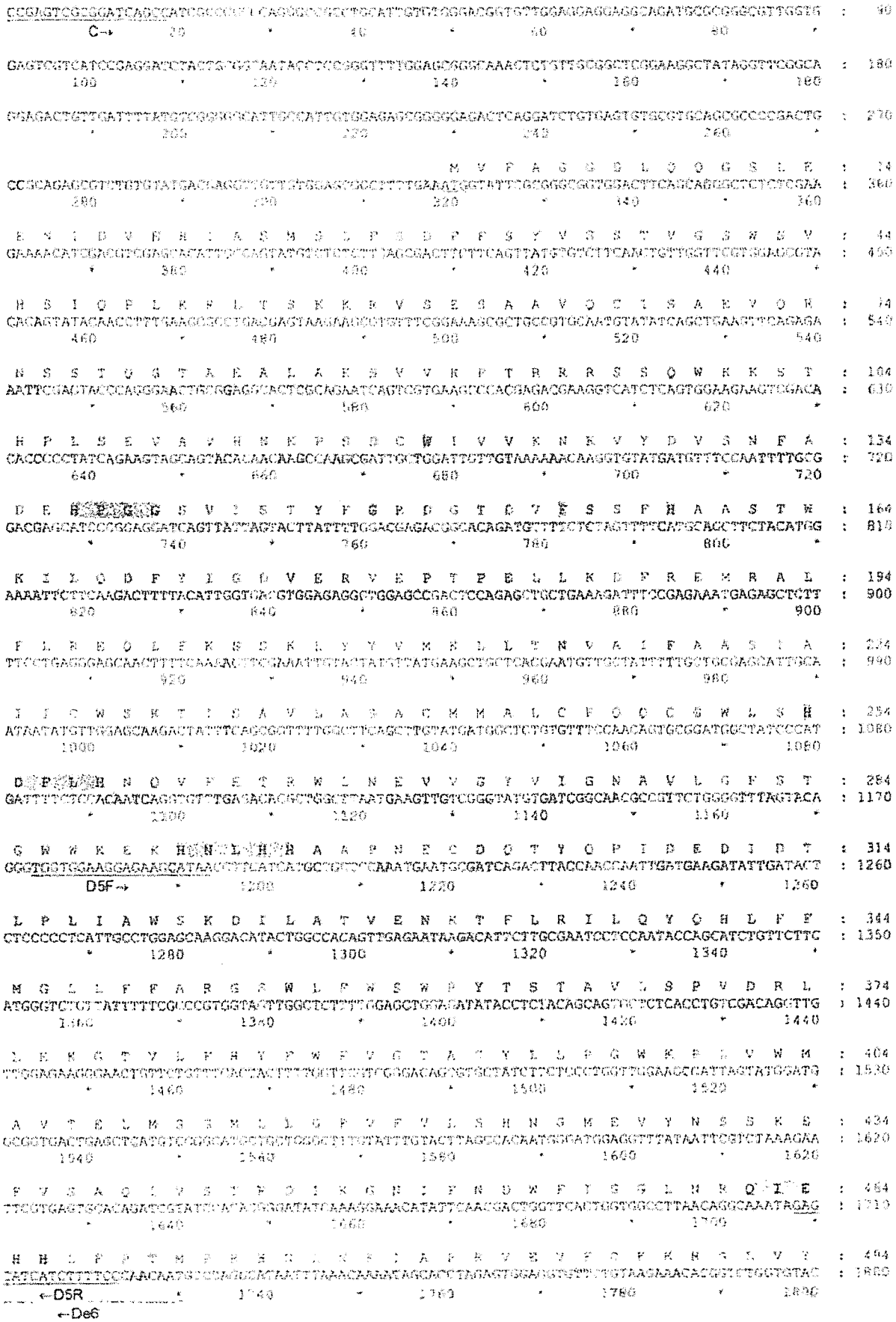


Fig. 3.1.1.1 Restriction map and sequencing strategy for the *PPDES6* cDNA. The figure shows the insert of the plasmid p*PPDES6*-cDNA with parts of the multiple cloning site (MCS) of pBluescript. The insert was designated *PPDES6* cDNA and is depicted as a black bar. The location and the orientation of the open reading frame (ORF) found is to be seen underneath. The upper scale indicates the length in base pairs (bp). Important cleavage sites are marked with vertical lines. The position of the PCR fragment B5pp employed as probe is marked over the cDNA. The sequencing strategy using restriction and deletions subclones is indicated by the bars depicted underneath (cf. 2.11). Doubtful sequence regions were verified by terminator sequencing with specific primers (arrows).


C→
D5F→
←D5R
←De6

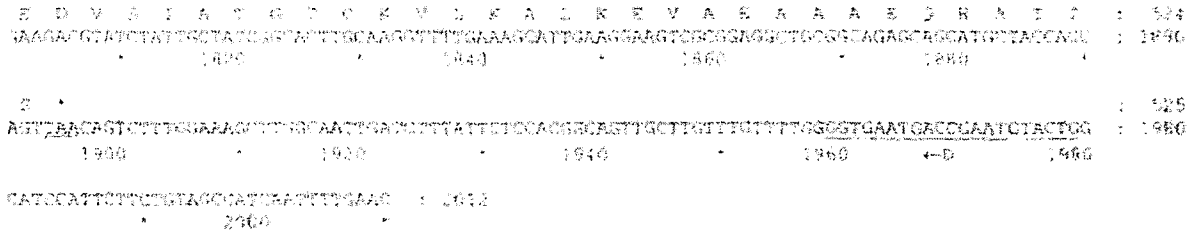


Fig. 3.1.1.2 Nucleotide sequence of the *PPDES6* cDNA with derived amino acid sequence. The start and stop codons of the open reading frame found are shown with wavy underlining. The underlined regions indicate the binding sites of the D5F/D5R (De6) primers which led to isolation of the cDNA (cf. Fig. 3.1.2.1), and the C/D primers which were employed to amplify the relevant genomic fragment *PPDES6* (Fig. 3.1.3.3). The eight invariant amino acids of the cytochrome b_5 family and the three histidine boxes of the desaturase domain are shown by shading. An incomplete cDNA clone which was identical in the overlap region to the *PPDES6* cDNA contained the extended 3' end 5'-AGTAAGGAGCCACGATTCGTCTCTGTTC-3' which was used to derive primer 4 (cf. Fig. 3.3.1).

3.1.2 Analysis of the primers which led to isolation of the *PPDES6* cDNA

In the original PCR screening with degenerate primers (Girke, 1995), the PCR fragment B5pp was isolated with the two primer combinations D5F/De6 and D5F/D5R. However, comparison of the sequences of the primers employed with their binding sites was not hitherto possible because primer sequences on the ends of PCR products cannot be utilized as sequence information. Now, with the *PPDES6* cDNA, the sequence of the putative binding sites is now available and can be compared with those of the primers.

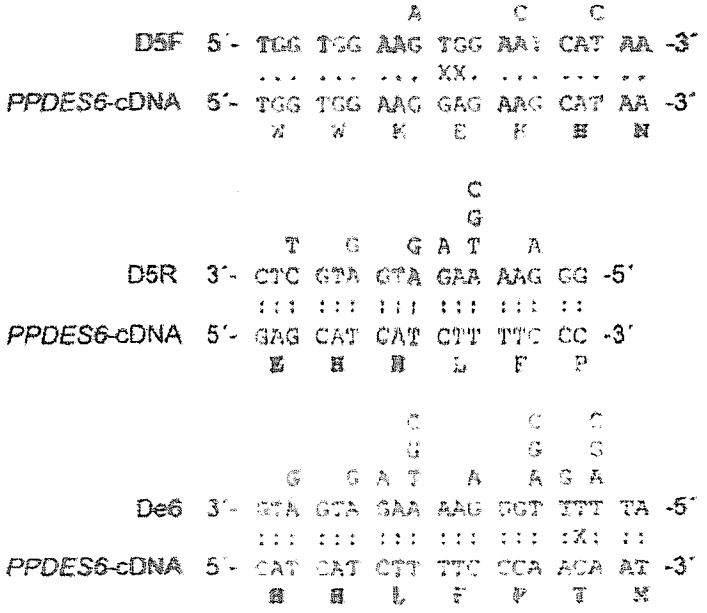


Fig. 3.1.2.1 Comparison of the sequences of the degenerate primers with the *PPDES6* cDNA. In the three alignments, the degenerate sequences of the primers D5F, D5R and De6 were arranged above the relevant regions of the PPDES6 cDNA (see Fig. 3.1.1.2). Complementary and identical bases are indicated respectively by colons (:) and dots (.), and base mismatches are indicated by an 'X'.

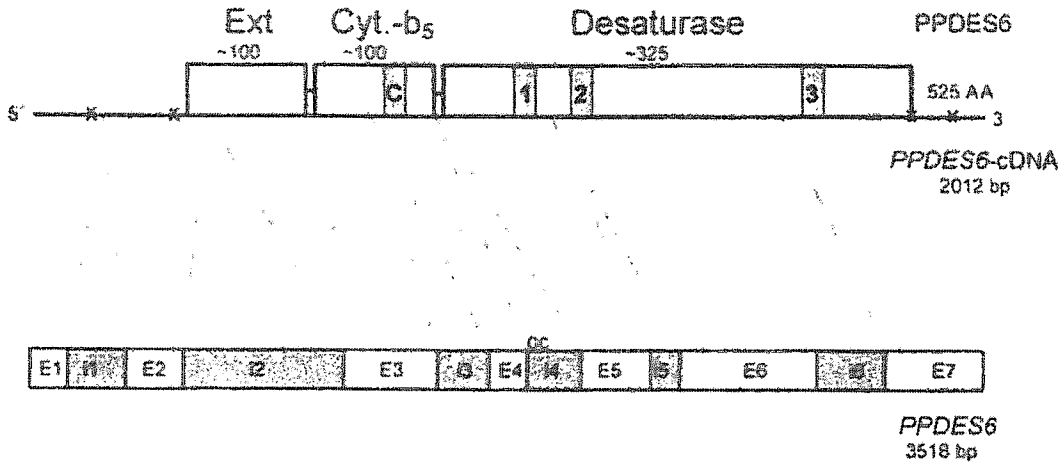


Fig. 3.1.4.1 Protein domains of PPDES6 with relevant cDNA and genomic fragment. The open reading frame found for the *PPDES6* cDNA (2012 bp) codes for the fusion protein PPDES6 (525 AA) which consists of three domains: an extension (Ext) of unknown function, a cytochrome b_5 domain (Cyt.-b_5) and a desaturase domain. Stop codons are depicted by crosses. The location of the HPGG box of the cytochrome b_5 is indicated by the gray region C and the three histidine boxes of the desaturase are indicated by regions 1-3. The location of introns i1-i6 and exons E1-E7 is indicated by thin lines connecting the cDNA and genomic sequence. *PPDES6* and the *PPDES6* cDNA are not depicted on the same scale.

To delimit the cytochrome b_5 domains, the sequence of the free cytochrome b_5 from *Nicotiana tabacum* was included in the alignment. The calculated identities between PPDES6 and the proteins in Table 3.1.4.1 are 11 to 21% of the whole lengths and 21 to 29% for the desaturase region. These are relatively low values for desaturases (Murata & Wada, 1995) because all desaturases known to date with identical regioselectivity from the same compartment have considerably higher identities, above 55%, which exist even between more remote organisms such as cyanobacteria and angiosperms. For example, six other PCR fragments from *P. patens* which were isolated in the same PCR screening as the fragment of PPDES6 (Girke, 1995) showed similarities of more than 60% to Δ12- and Δ15-desaturases from cyanobacteria and angiosperms.

In order to compare the position of PPDES6 with remotely related desaturases, a substantially more complex sequence alignment was performed using only the desaturase regions between the first and third histidine boxes (from the last amino acid of the first box to the first amino acid of the third box, Fig. 6.1). The regions on the other sides of the histidine boxes were ignored in this case in order to avoid the desired comparisons of similarity being falsified by existing differences in length. Various representatives of the Δ9-, Δ12- and Δ15-desaturases were used for the alignment, also including the six other fragments from *P. patens* mentioned above.

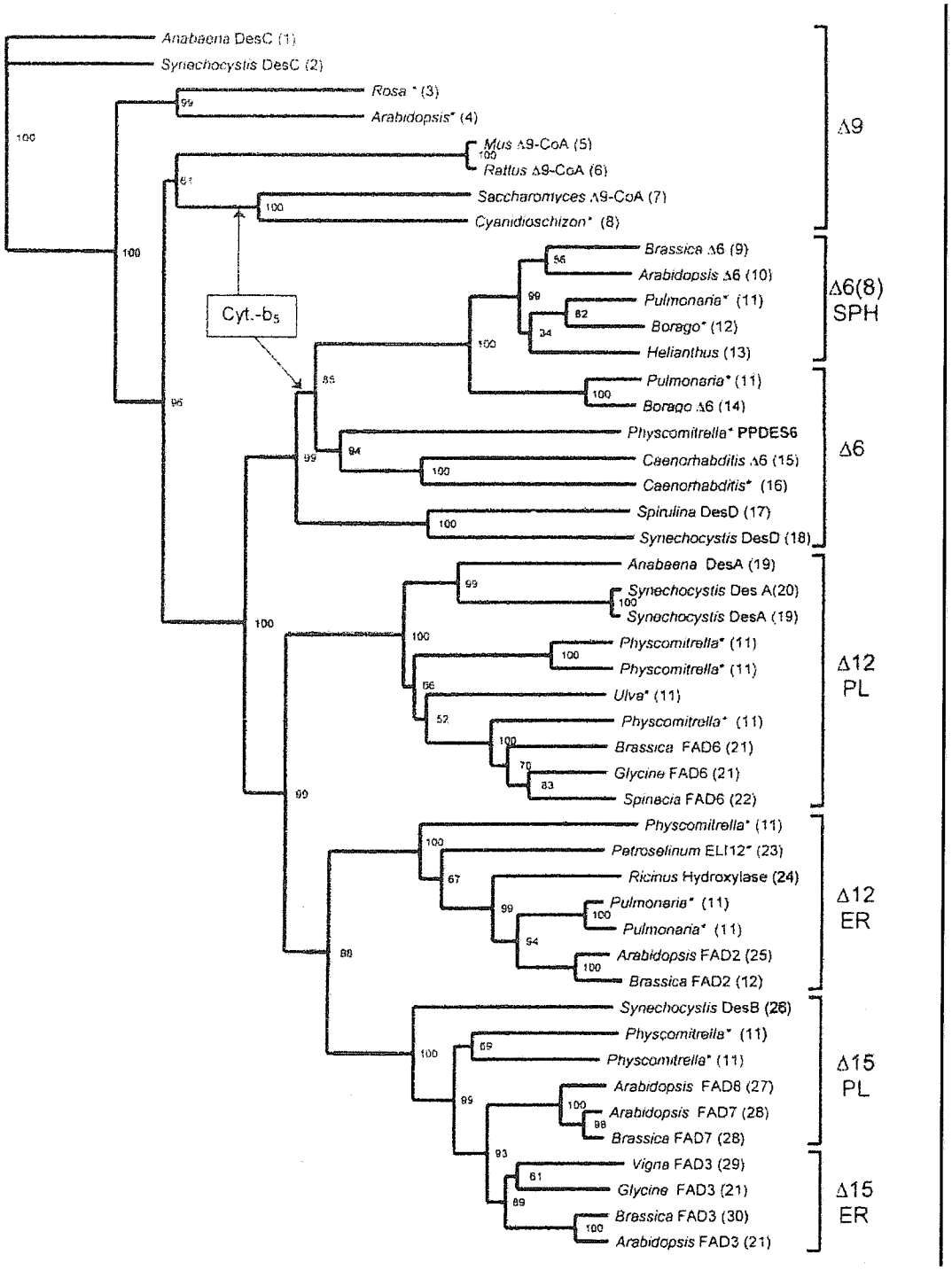


Fig. 3.1.4.3 Phylogram calculated from an amino acid alignment of functionally different desaturases. The regioselectivities and the subcellular location (plastids: PL and endoplasmic reticulum: ER) of the desaturases are indicated in the right-hand margin.

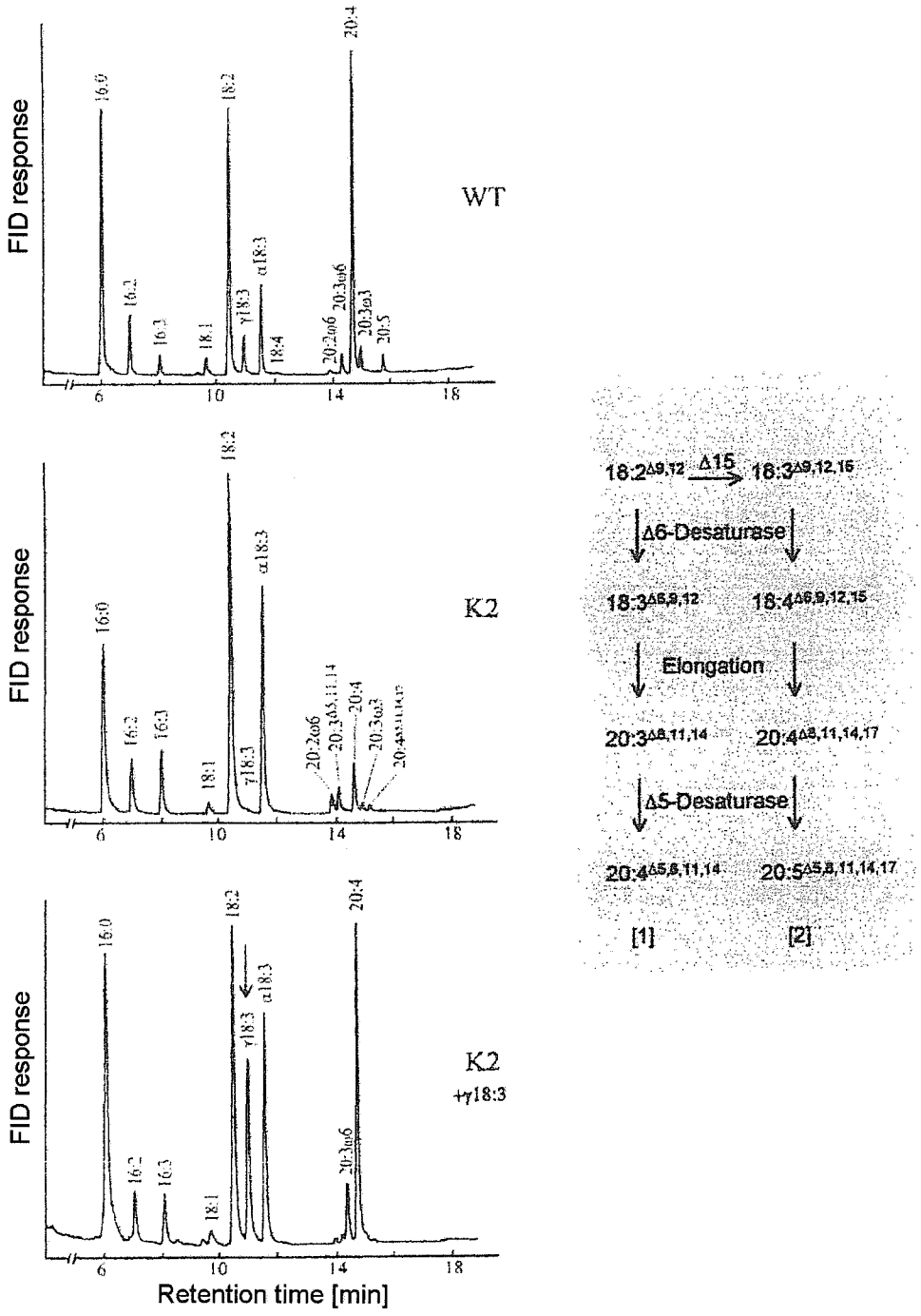


Fig. 3.4.1 Fatty acid profiles of the *P. patens* wild type and of the K2 mutant. The fatty acid methyl esters (FAME) of the total lipids of 14-day old liquid cultures were analyzed by GLC. The profile of the wild type (WT) is indicated top, and that of the mutant (K2) underneath. The lowest chromatogram shows the fatty acid pattern of a K2 culture which was grown under identical conditions but contained 50 µm γ18:3 ($18:3^{\Delta 6,9,12}$). The percentage areas of the peaks are indicated in Figures 3.4.2 and 3.4.3. The biosynthetic pathways for $20:4^{\Delta 5,8,11,14}$ and $20:5^{\Delta 5,8,11,14,17}$ are depicted in the gray box.

3.5 Functional expression of the *PPDES6* cDNA in *Saccharomyces cerevisiae*

In order to be able to preclude with certainty that the fatty acid phenotype of the *P. patens* mutants (K1-K2) is not attributable to loss of a regulatory gene product for a Δ6-desaturase, the *PPDES6*-cDNA was functionally expressed in the *Saccharomyces cerevisiae*. For this purpose, the vectors pYES2 and pYESΔ6 described in section 2.12.2 were transformed into the *S. cerevisiae* strain INVSC1. The approach with pYESΔ6 represented the construct for expressing the desaturase, and pYES2 a vector without insert control. In the cloning of pYESΔ6, the coding region of the cDNA was cutout of the vector pSK (Fig. 3.2.2) as *Kpn* I/*Xba* I fragment and inserted downstream of the galactose-inducible GAL1 promoter of pYES2. For the expression experiments, the transformed cell clones were grown in minimal medium and the promoter was induced with galactose in the early logarithmic phase. In some cases, expression took place in the presence of the fatty acids $18{:}2^{\Delta 9,12}$ and $18{:}3^{\Delta 9,12,15}$ which represent potential substrates of a Δ6-desaturase and are not provided by *S. cerevisiae* owing to the absence of desaturases. The minimal medium used contained the detergent Tergitol NP-40 to solubilize these fatty acids (section 2.18). The fatty acids were applied at the same time as the promoter was induced. After culturing for a further four to five generation times, the induced cells were harvested and their total fatty acids were analyzed by GLC as in preceding section 3.4. The fatty acid profiles determined in this way are depicted in Figure 3.5.1 and Table 3.5.1.

The *S. cerevisiae* cells which contained the empty pYES2 vector show the very simple fatty acid pattern of the wild type, which is composed of saturated and monounsaturated C16 and C18 fatty acids (Kajiwara *et al.*, 1996; Wagner & Paltauf, 1994). In the presence of linoleic acid there is a marked incorporation of the added fatty acid into the lipids. After addition of 0.1 mM linoleic acid the proportion of linoleic acid is about 20% of the total fatty acids. In contrast, synthesis of new fatty acids is to be found with the *S. cerevisiae* cells transformed with the expression construct pYESΔ6. After addition of $18{:}2^{\Delta 9,12}$ there was formation of $18{:}3^{\Delta 6,9,12}$, and in the presence of $18{:}3^{\Delta 9,12,15}$ there was production of $18{:}4^{\Delta 6,9,12,15}$. Surprisingly, it was also possible to detect the diunsaturated fatty acids $16{:}2^{\Delta 6,9}$ and $18{:}2^{\Delta 6,9}$, as was the case especially in the absence of external fatty acids and to a smaller extent also on addition of $18{:}2^{\Delta 9,12}$. All four newly formed fatty acids had a Δ6 double bond whose position could be confirmed by GLC-MS analyses of the corresponding fatty acid pyrrolidides. This new formation of Δ6 unsaturated fatty acids in the cells transformed with pYESΔ6 unambiguously proves that the *PPDES6* cDNA codes for a Δ6-desaturase. The Δ6-desaturase conversions were highest after addition of $18{:}2^{\Delta 9,12}$, with a maximum of 7.4% $18{:}3^{\Delta 6,9,12}$. By contrast, the maximum reached in the presence of $18{:}3^{\Delta 9,12,15}$ was 3% $18{:}4^{\Delta 6,9,12,15}$. This difference in quality indicates that 18:2 is the preferred substrate of the desaturase. This preference is consistent with the fact that PPDES6 is involved in the formation of arachidonic acid in *P. patens*, the synthesis of which proceeds via a Δ6-desaturation of $18{:}2^{\Delta 9,12}$ (Fig. 3.4.1).

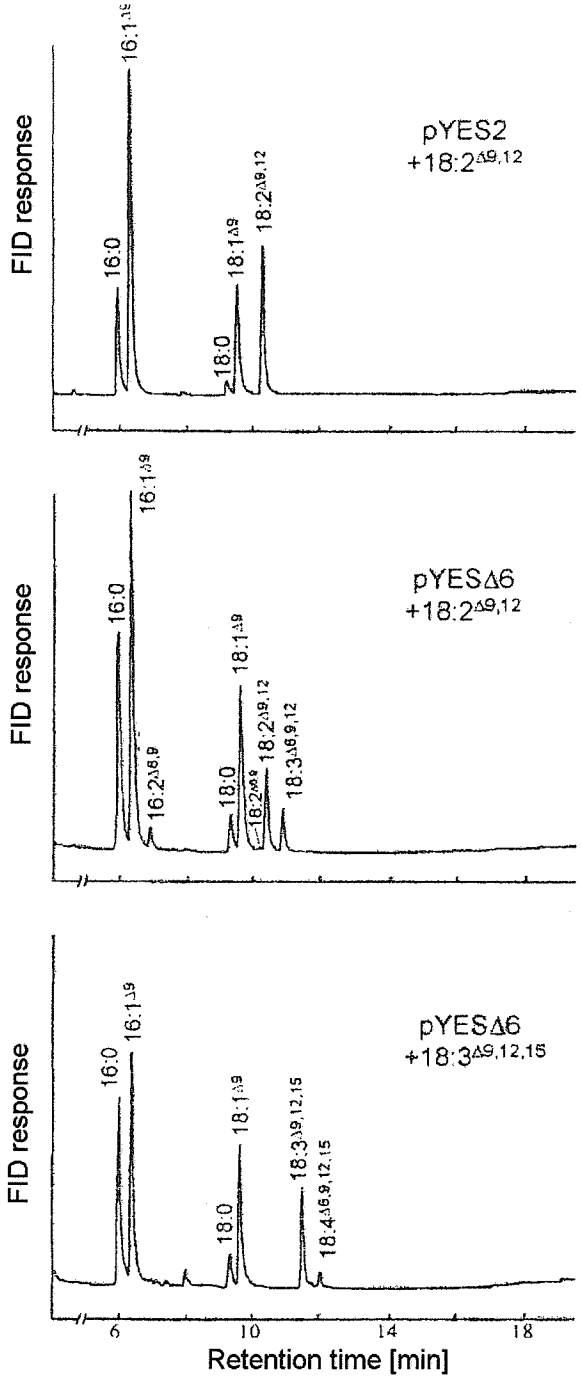


Fig. 3.5.1 Fatty acid profiles of transformed *S. cerevisiae* cells. The fatty acid methyl esters of the total lipids of INVSC1 cells transformed either with pYES2 (wild-type control) or pYESΔ6 (*PPDES6* cDNA) were analyzed by GLC. The cells were grown in minimal medium (CMdum) at 30°C. The relevant percentage areas of the signals are indicated in columns 4, 6 and 8 in Table 3.5.1. The two upper chromatograms show fatty acid profiles after addition of 0.1 mM $18:2^{\Delta 9,12}$ (18:2), and the lowest one shows the profile after addition of $18:3^{\Delta 9,12,15}$ (α18:3).

Fatty acid proportions in yeast							
Fatty acids	pYES2			pYESΔ6			
	-	18:2		-	18:2		α18:3
		1.0 mM	0.1 mM		0.1 mM		0.1 mM
16:0	16.4	24.5	14.6	16.1	23.8	25.8	25.8
$16:1^{\Delta9}$	54.0	3.9	43.3	55.5	38.1	16.8	31.4
$16:2^{\Delta6,9}$	-	-	-	4.2	1.7	0.1	-
18:0	3.2	4.8	2.4	2.4	4.0	4.2	4.7
$18:1^{\Delta9}$	24.9	1.8	17.0	19.7	19.1	8.0	19.2
$18:2^{\Delta6,9}$	-	-	-	0.6	0.2	-	-
$18:2^{\Delta9,12}$	-	61.4	20.9	-	8.5	36.0	-
$18:3^{\Delta6,9,12}$	-	-	-	-	4.0	7.4	-
$18:3^{\Delta9,12,15}$	-	-	-	-	-	-	11.7
$18:4^{\Delta6,9,12,15}$	-	-	-	-	-	-	3.0

Tab. 3.5.1 Expression of the *PPDES6* cDNA in *S. cerevisiae.* The fatty acid methyl esters of the total lipids of cells (INVSC1) transformed with pYES2 (wild-type control) or with pYESΔ6 (*PPDES6* cDNA) were analyzed by GLC and indicated here in percentage area. The cells were cultured in minimal medium (CMdum) at 30°C. The promoter was induced at an OD_{600} of 0.25 with 2% galactose, harvesting after a further four generations. In some cultures, the fatty acids $18:2^{\Delta9,12}$ (18:2) or $18:3^{\Delta9,12,15}$ (α18:3) were added in the stated concentration at the time of induction. Two independent analyses are shown for pYESΔ6 with 18:2, the first example reproducing the data for the middle chromatogram in Figure 3.5.1.

Total proteins were extracted from cells transformed with pYESΔ6 or pYES2 at various times after the galactose induction and were fractionated over an SDS polyacrylamide gel (results not shown). In the gel stained with coomassie brilliant blue, no additional protein bands which might indicate accumulation of the Δ6-desaturase were evident with pYESΔ6.

An unexpected property of the Δ6-desaturase is the formation of $16:2^{\Delta6,9}$ and $18:2^{\Delta6,9}$, because these fatty acids are undetectable in *P. patens*. The explanation for their occurrence is that the putative precursors $16:1^{\Delta9}$ and $18:1^{\Delta9}$ are produced in large quantities by *S. cerevisiae*. By contrast, in *P. patens* $16:1^{\Delta9}$ is undetectable and $18:1^{\Delta9}$ is detectable only in very small amounts. Moreover, the Δ6-desaturase preference is probably for 16:1 over 18:1, because distinctly more $16:2^{\Delta6,9}$ (4.2%) than $18:2^{\Delta6,9}$ (< 1%) is formed.

4 Summary

The moss *Physcomitrella patens* contains large quantities of arachidonic acid (20:4) in its membrane lipids, and the biosynthesis thereof from linoleic acid (18:2) via desaturations and elongation requires Δ6- and Δ5-desaturases which have not previously been cloned. To isolate one of these desaturases it was possible in the work for my diploma to isolate by a PCR approach with degenerate primers a cDNA fragment with few similarities to acyl lipid desaturases. The intention in the work presented here was to isolate, sequence and functionally identify the corresponding complete cDNA clone.

The complete cDNA was isolated from a *P. patens* cDNA library and sequenced as *PPDES6* cDNA with a length of 2012 bp. The amino acid sequence PPDES6 derived therefrom contains 525 AA (59.3 kD). Sequence comparisons with databases revealed that PPDES6 has three domains: an N-terminal segment of about 100 AA without similarities to known sequences, a middle cytochrome b_5 domain and a C-terminal sequence with small similarities (29%) with acyl lipid desaturases. The relevant genomic fragment (*PPDES6*) was isolated by PCR. It contains six introns, with the 4th intron being inserted directly behind the triplet after which the similarity to cytochrome b_5 sequences ceases. Intron 4 additionally has the unusual 5' GC splice boundary.

Various strategies were followed to elucidate the function of PPDES6. One obvious way was to modulate the enzyme activity by transformation with sense and antisense constructs. In addition, the gene disruption method, which has not to date become established in plant molecular biology, was employed to enable targeted and complete disruption of a gene. For this purpose, *P. patens* was transformed with the linear genomic sequence of the desaturase into which a positive selection marker had been inserted. After seven transformation experiments in each case it was not possible to isolate any stable transformants in the sense and antisense approaches, and this negative result is inexplicable at present. On the other hand, the gene disruption approach is provided with 56 mutants reproducible in large numbers of transformants. The PCR and Southern blot analyses of five randomly selected transgenic lines after disruption revealed that the insertion construct had been inserted into the *PPDES6* gene in all five cases. Expression of the *PPDES6* gene which shows a very high level of transcription in the wild type was no longer detectable in a Northern blot. Fatty acid analyses of the generated insertion mutants revealed a marked increase in linoleic acid and an almost complete decline in γ-linolenic acid ($18:3^{\Delta6,9,12}$), arachidonic acid ($20:4^{\Delta5,8,11,14}$) and eicosapentaenoic acid ($20:5^{\Delta5,8,11,14,17}$). Complementation of this biochemical phenotype was possible by γ-linolenic acid feeding. These analyses show that the mutants had lost not the Δ5-desaturase but a Δ6-desaturase which transforms $18:2^{\Delta9,12}$ into $18:3^{\Delta6,9,12}$ and $18:3^{\Delta9,12,15}$ into $18:4^{\Delta6,9,12,15}$. It was possible to verify this function by expression of the *PPDES6* cDNA in *Saccharomyces cerevisiae*, because only the transformed yeast cells were able to introduce a further double bond in the

Δ6 position in unsaturated fatty acids. Furthermore, the Δ6 desaturation of $16:1^{\Delta 9}$ in yeast indicated that PPDES6 is a Δ- and not an ω-desaturase. A further important effect in the case of the insertion mutants was the decline in the total of C20 fatty acids. It indicates that the relevant elongation system is specific for γ-linolenic acid in *P. patens*. In addition, the loss of 20:4 in the plastidic and microsomal lipids indicates that the Δ6-desaturase is localized in the ER, and the presence of the cytochrome b_5 fusion provides support for this.

It was possible in this work for the first time, as far as is known, to disrupt an unknown gene of a multicellular plant in a targeted manner by homologous recombination and to characterize its function through the resulting biochemical phenotype. In addition, the generated transgenic lines represent the first plant mutants with a disrupted Δ6-desaturase gene about whose identity there is no doubt owing to the expression in yeast.

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